Sympathetic Activation Causes Focal Adhesion Signaling Alteration in Early Compensated Volume Overload Attributable to Isolated Mitral Regurgitation in the Dog

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Abstract—We reported that left ventricular (LV) dilatation after 4 weeks of isolated mitral regurgitation (MR) in the dogs is marked by extracellular matrix loss and an increase in adrenergic drive. Given that extracellular matrix proteins and their receptor integrins influence β-adrenergic receptor (β-AR) responses in vitro, we tested whether β1-AR activation modulates focal adhesion (FA) signaling and LV remodeling in these same dogs with isolated MR. Normal dogs were compared with dogs with MR of a 4-week duration and with MR dogs treated with β1-RB blockade (β1-RB) (extended-release metoprolol succinate, 100 mg QD) that was started 24 hours after MR induction. In MR LVs, a decrease in collagen accumulation compared with normal dogs was associated with a decrease in FA kinase tyrosine phosphorylation, along with FA kinase interaction with adapter and cytoskeletal proteins, p130Cas and paxillin, respectively, as determined by immunoprecipitation assays. There was increased phosphorylation of stress related molecules p38 mitogen-activated protein kinase (MAPK) and Hsp27 and survival signaling kinases extracellular signal-regulated kinase 1/2 and AKT, with no evidence of cardiomyocyte apoptosis. β1-RB attenuated FA signaling loss and prevented p38 MAPK, Hsp27, and AKT phosphorylation induced by MR and significantly increased LV epicardial collagen content. However, β1-RB did not improve LV endocardial collagen loss or LV dilatation induced by MR. Isolated myocytes from normal and MR dog hearts treated with β1- or β2-AR agonists demonstrated no difference in FA kinase, p38 MAPK, Hsp27, or AKT phosphorylation. These results showed that chronic stimulation of β1-AR during early compensated MR impairs FA signaling that may affect myocyte/fibroblast–extracellular matrix scaffolding necessary for LV remodeling. (Circ Res. 2008;102:1127-1136.)

Key Words: β-adrenergic receptors ▪ focal adhesion ▪ volume overload ▪ mitral regurgitation ▪ apoptosis

Myocardial remodeling in response to pressure or volume overload (VO) is an adaptation of myocardial structure to accommodate chronic changes in myocardial demand. This remodeling is thought ultimately to drive a maladaptive process of ventricular dilatation and pump dysfunction that contributes to the pathogenesis of heart failure. In contrast to pressure overload, the VO of isolated mitral regurgitation (MR) results in left ventricular (LV) dilatation, side-to-side slippage of cells, and degradation of extracellular matrix (ECM) proteins. In addition to these structural changes, there is development of cardiac hypertrophy and subsequent LV dysfunction in MR that are improved by β-adrenergic receptor blockade (β-RB) but not by angiotensin-converting enzyme inhibition or angiotensin type I receptor blockade, suggesting that the adrenergic system is more central to the pathophysiology of VO of isolated MR. Moreover, studies in humans and dogs have demonstrated an increase in adrenergic drive even in a mild compensated state of isolated MR, most likely attributable to the early achievement of preload reserve.

β-Adrenergic receptors (β-ARs) belong to the large family of G protein–coupled receptors that is involved in positive inotropic, chronotropic, and lusitropic responses through activation of G protein. In the failing hearts, several defects in β-AR signaling have been detected, including receptor downregulation and uncoupling from the stimulatory G protein, increased level of inhibitory G protein subunits, decreased adenyly cyclase activity, and increased β-AR kinase-1 expression and activity. The ability of integrins as ECM receptors to regulate cytoskeletal architecture has been well characterized in vitro.
and in vivo. In addition, integrin signaling has been implicated in G protein–coupled receptor–, hormone–, and growth factor–induced alterations in gene transcription in cardiac myocytes. Integrons are a family of heterodimeric transmembrane receptors (composed of α and β subunits) containing extracellular ligand binding domains that show binding specificity for ECM components and a short cytoplasmic domain that serves to couple integrins with the actin cytoskeleton. Binding of a matrix protein to an integrin heterodimer typically results in the activation of the nonreceptor tyrosine kinase, focal adhesion kinase (FAK). Activated FAK, in turn, recruits the nonreceptor tyrosine kinase c-Src, the multifunctional adapter molecule Grb2, p130Cas, and FAK knockout result in defective heart development and early embryonic lethality. Interestingly, in mice with myocyte-restricted FAK inactivation, an eccentric cardiac hypertrophy develops with age and in response to pressure overload stimuli, suggesting that downregulation of focal adhesion (FA) signaling is associated with the development of eccentric cardiac hypertrophy. In a similar study, persistent challenge of mice with myocyte-restricted FAK inactivation leads to enhanced cardiac fibrosis and cardiac dysfunction in comparison with challenged genetic controls.

Pressure overload–induced cardiac hypertrophy is accompanied by enhanced expression of ECM proteins, integrins, and enhanced activation of FA signaling that correlates with advancement of hypertrophy. However, little is known about integrin signaling during VO-induced cardiac hypertrophy that is associated with a decrease in ECM accumulation as during isolated MR in the dog. We reported an increase in catecholamine release into the LV interstitial fluid space after about 4 weeks of MR. We extended this work using samples from these same dogs and now show for the first time that catecholamine release into the LV interstitial fluid space after approximately 24 hours after the last dose of metoprolol and, thus, may not reflect the effect of β1-RB on LV remodeling in the VO of isolated MR.

### Materials and Methods

Mitral valve regurgitation was induced in conditioned mongrel dogs of either sex (19 to 26 kg) by chordal rupture using a fluoroscopic guided catheterization method previously described in our laboratory. Dogs were randomly assigned to 1 of 5 groups: (1) unoperated controls (n=6); (2) 2 and 4 weeks of MR (2W-MR and 4W-MR; n=6); and (3) 2 and 4 weeks of MR treated with β1-AR blocker (extended-release metoprolol succinate, 100 mg PO, once daily; n=6) starting 24 hours after MR induction. In these same animals, we have previously reported the results of M-mode echocardiography, which was performed in the conscious state at baseline and at the time of euthanasia. Animals were maintained at a deep plane of general anesthesia using isoflurane and were mechanically ventilated. At the end of the in vivo experiments, the heart was arrested with intracardiac injection of KCl and quickly exsanguinated and placed in phosphate-buffered saline solution. A portion of the LV (midmyocardium) was cut and snap-frozen in liquid nitrogen for subsequent biochemical analyses. This study was approved by the Animal Services Committees at the University of Alabama at Birmingham and Auburn University.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

### Results

#### Effects of β1-RB on LV Remodeling and Function Following MR

Mean arterial pressure was significantly decreased compared with controls in both 4W-MR and 4W-MR+β1-RB dogs (Table). Heart rate, LV, end-diastolic pressure, and mean systemic vascular resistance did not differ from controls in 4W-MR and 4W-MR+β1-RB dogs. However, cardiac output was significantly decreased in 4W-MR+β1-RB dogs compared with controls. There was a trend toward increases in LV mass in 4W-MR and 4W-MR+β1-RB dogs compared with controls, but this did not achieve statistical significance. We have previously shown in these same 4W-MR dogs a marked increase in catecholamine release into the LV interstitial fluid space that was significantly attenuated by β1-RB. The resting heart rate was measured under general anesthesia approximately 24 hours after the last dose of metoprolol and, thus, may not reflect the effect of β1-RB on heart rate in the conscious state. However, in these same dogs, heart rate response to stellate stimulation was decreased in 4W-MR+β1-RB compared with 4W-MR dogs, suggestive of a negative chronotropic effect of β1-RB.

LV end-diastolic dimension increased similarly in both 4W-MR and 4W-MR+β1-RB compared with baseline (Table). In addition, cardiac function assessed by LV fractional

| Table. LV Hemodynamics and Echocardiography in Control, 4W-MR, and 4W-MR+β1-RB Dogs |
|--------------------------------------|----------------|----------------|----------------|
| Control (n=6) | 4W-MR (n=6) | 4W-MR+β1-RB (n=6) |
| Heart rate, bpm | 94±6 | 86±6 | 83±5 |
| Mean arterial pressure, mm Hg | 84±3 | 65±4* | 62±3* |
| LV end diastolic pressure, mm Hg | 4±1 | 8±1 | 6±2 |
| Systemic vascular resistance, dyne · sec · cm⁻¹ | 2007±219 | 1606±240 | 2342±176 |
| Cardiac output, L/min | 3.6±0.4 | 3.8±0.6 | 2.1±0.1* |
| LV mass, g/kg | 4.4±0.2 | 5.0±0.3 | 5.0±0.3 |
| LV end-diastolic dimension, % change | 9.2±2.9† | 17.7±4.7† |
| LV end-systolic dimension, % change | -7.4±5.8 | 0.1±3.6 |
| LV end-diastolic wall thickness, % change | -12.0±8.5 | -10.2±5.8 |
| Wall thickness/end-diastolic dimension, % change | -18.6±9.3† | -20.9±6.5† |
| Fractional shortening, % change | 32.7±11.3† | 34.3±13.9† |

Values presented as means±SEM. *P<0.05 vs control, †P<0.05, % change vs baseline study.
shortening was significantly increased from baseline in the 4W-MR group, because LV end-systolic dimension remained unchanged (Table). However, this increase was not affected by β1-RB. In a similar fashion, response of LV dP/dt to stellate stimulation was similar in 4W-MR and 4W-MR+β1-RB dogs, whereas stellate-stimulated heart rate change was attenuated in 4W-MR+β1-RB compared with control and 4W-MR dogs. There was a significant decrease in LV end-diastolic wall thickness decreased significantly in 4W-MR group compared with baseline, along with a significant increase in LV end-diastolic dimension, indicating eccentric LV remodeling. However, these changes were not affected by β1-RB treatment.

**β1-RB Reduces Interstitial Collagen Degradation Induced by MR**

Quantitative evaluation of myocardial interstitial collagen revealed a significant decrease in volume percentage of collagen for untreated 4W-MR hearts compared with controls at both the endocardium and epicardium levels (Figure 1). Moreover, the decrease in collagen accumulation was evident at the endocardium at 2 weeks after MR (Figure 1 in the online data supplement) and was sustained for 4 months after MR (data not shown). β1-RB prevented collagen loss by ~68% in the epicardium induced by 4 weeks of MR but has no detectable effect on interstitial endocardium collagen.

**β1-RB Prevents FAK Signaling Downregulation Induced by MR**

Several studies have shown that pressure overload–induced cardiac hypertrophy is associated with an increase in integrin signaling. However, these models of cardiac hypertrophy were associated with an increase rather than a decrease in ECM deposition. Because maximal cardiac hypertrophy requires ECM, we examined whether components of the integrin-signaling cascade were altered after 4W-MR. We examined tyrosine phosphorylation of FAK and correlated these with ECM changes. In control dogs, there was basal FAK tyrosine phosphorylation as determined by blotting with anti-phosphotyrosine antibodies (Figure 2A). MR induction for 4 weeks led to a significant decrease in tyrosine phosphorylation of FAK without a decrease in FAK expression, because the same blot rebotted with anti-FAK antibodies

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** β1-RB prevents FAK tyrosine downregulation induced by MR. A, LV extracts from control, 4W-MR, and 4W-MR+β1-RB dogs were immunoprecipitated (IP) with anti-FAK antibodies and immunoblotted with anti-phosphotyrosine antibodies. Top, Representative autoradiogram (with each lane from a single gel exposed for the same duration). Bottom, Fold induction (n=6 each group). *P<0.05, 4W-MR vs control; #P<0.05, 4W-MR+β1-RB vs 4W-MR. B, Representative immunoblots showing accumulation of phospho-FAK Tyr397, -576/577, -861, and -925 in LV extracts from control, 4W-MR, and 4W-MR+β1-RB. Blots were stripped and blotted with anti-FAK antibodies.
showed equal FAK expression levels between control and MR dogs (Figure 2A). A similar decrease in FAK tyrosine phosphorylation was observed at 2 weeks after MR (supplemental Figure II). The lack of quantitative changes in total FAK expression between control and MR hearts was also associated with a lack of detectable changes in FAK distribution, as evaluated by double immunostaining using anti-FAK polyclonal antibodies (supplemental Figure III). FAK immunolabeling was localized throughout the myocardium of control hearts, including the cardiomyocytes that also stained positively for sarcomeric α-actin, and some staining of the interstitial space. There was also FAK staining of endothelial cells and the medial layer of blood vessels (data not shown).

In 4W-MR hearts, there was no qualitative change in FAK immunolabeling compared with normal hearts. Thus, FAK expression and distribution were not significantly affected by 4W-MR. Treatment with β1-RB significantly reduced the decrease in FAK tyrosine phosphorylation induced by MR at 4 weeks but did not reach statistical significance in 2W-MR dogs compared with controls (Figure 2A and supplemental Figure II). Because FAK can be tyrosine-phosphorylated on a number of tyrosine residues, including Tyr397, -578/577, -861, and -925 in response to various stimuli, we next mapped the phosphorylation site on FAK using series of well-characterized phospho-specific antibodies.24 FAK-Tyr397, -578/577, -861, and -925 phosphorylation decreased significantly in 4W-MR dogs compared with controls and treatment with β1-RB improved the decrease in FAK-Tyr397, -578/577, -861 tyrosine phosphorylation sites without any detectable differences between these different sites. However, β1-RB was without significant effect on FAK-Tyr925 phosphorylation, suggesting a different mechanism of regulation of this FAK tyrosine phosphorylation site (Figure 2B).

Consistent with a reduction in FAK tyrosine phosphorylation, there was a decreased association between FAK and p130CAS, and FAK and paxillin, 2 important components of FAK complex that associate with and are phosphorylated by FAK,15,16 in 4W-MR dogs compared with controls (Figure 3A). Treatment with β1-RB prevented both FAK–paxillin and FAK-p130CAS dissociation. To investigate whether p130CAS and paxillin are also affected by 4W-MR, analysis of VO of isolated MR differentially regulates the activation of these 3 MAP-kinases with p38 MAPK and ERK1/2 being activated in 4A and 4B and supplemental Figure V). This suggests that the VO of isolated MR differentially regulates the activation of these 3 MAP-kinases with p38 MAPK and ERK1/2 being activated in response to MR and that p38 MAPK is dependent on β1-AR stimulation.

MR-Induced FA Signaling Alteration Is Not Associated With Cell Death by Apoptosis

Both loss of ECM and/or loss of FAK signaling have been shown to lead to cell death termed anokies.28–30 To examine whether apoptosis occurs after ECM loss and subsequent FA alteration during induction of MR, we measured caspase-3 activity and DNA fragmentation by ELISA and TUNEL assay in control, 4W-MR, and 4W-MR + β1-RB groups (Figure 5). We did not detect any difference among control, 4W-MR, and 4W-MR + β1-RB in either caspase-3 activity (normal, 249 ± 13 relative fluorescence unit (RFU)/min per milligram protein; 4W-MR, 242 ± 27 RFU/min per milligram protein; 4W-MR + β1-RB, 198 ± 26 RFU/min per milligram protein), DNA fragmentation (normal, 5.2 ± 0.9 optical density (OD)410 to OD500/mg protein; 4W-MR, 5.8 ± 1.4 OD410 to OD500/mg protein; 4W-MR + β1-RB, 4.00 ± 0.9 OD410 to OD500/mg protein), or the percentage of TUNEL-positive cardiomyocytes (normal, 0.026 ± 0.011%; 4W-MR, 0.021 ± 0.002%; 4W-MR + β1-RB, 0.03 ± 0.015%). These data showed that MR at this early compensatory hypertrophy stage is not associated with myocyte apoptosis despite a loss of ECM and FA signaling.

MR-Induced AKT and HSP27 Phosphorylation Is Prevented by β1-RB

The absence of apoptotic markers in MR hearts despite the decrease in FA signaling led us to hypothesize that other compensatory signaling molecules may be activated to counteract the effect of ECM loss. One of the molecules that has been shown to play a role in survival and protection of myocytes against apoptosis is the phosphatidylinositol 3-kinase/AKT pathway.31 As FAK has been identified as the major site for binding of phosphatidylinositol 3-kinase, the inositol lipid products of which are key mediators of Akt activation,32 we next examined whether induction of MR is associated with an increase in AKT phosphorylation and whether β1-RB prevents this activation. Immuno blotting with anti–phospho-AKT at the Ser473 residue, which has been shown to be required for maximal activation of

regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways, each of which has been implicated in hypertrophic signal transduction.25–27 Therefore, we examined whether the loss of FA signaling observed in MR dogs was associated with changes in p38 MAPK, ERK1/2, and JNK phosphorylation by immunoblot analysis. Control animals presented basal p38 MAPK and ERK1/2 phosphorylation and induction of MR for 4 weeks led to an increase in p38 MAPK and ERK1/2 phosphorylation (Figure 4A and 4B). However, 4 weeks of MR had no detectable effect on JNK phosphorylation state (Figure 4C). A similar increase in p38 MAPK and ERK1/2 phosphorylation was also observed in the 2W-MR group (supplemental Figure V and data not shown). Interestingly, β1-RB completely abolished p38 MAPK phosphorylation induced after 4W-MR, whereas activation of ERK1/2 was not significantly affected at 2 and 4 weeks after MR (Figure 4A and 4B and supplemental Figure V). This suggests that the VO of isolated MR differentially regulates the activation of these 3 MAP-kinases with p38 MAPK and ERK1/2 being activated in response to MR and that p38 MAPK is dependent on β1-AR stimulation.

β1-RB Prevents Alteration of FAK Downstream Signaling Induced by MR

Putative signaling pathways downstream from FAK/p130CAS/ paxillin could involve activation of the extracellular signal-
AKT showed an increase in AKT phosphorylation following 4W-MR (Figure 6A). The amount of total Akt expression was not different between the 2 groups of animals. The increase in AKT phosphorylation was abrogated in 4W-MR/H11001/H92521-RB dogs, suggesting that 1-AR stimulation mediates AKT activation. Interestingly, these data also showed that FAK and AKT phosphorylation are differentially regulated by 1-ARs in the MR model, with AKT phosphorylation being independent from FAK activation.

Another molecule that has been shown to be downstream of p38 MAPK and to confer protection and antiapoptotic properties for the heart is heat shock protein (Hsp)27. MR induction for 4 weeks increased Hsp27 phosphorylation, as assessed by immunoblot (Figure 6B). As for p38 MAPK activation, 1-RB abolished Hsp27 activation induced by 4W-MR (Figure 6B). These data, together, suggest that stimulation of 1-AR is involved in the activation of antiapoptotic pathways, AKT and Hsp27, that may compensate for the loss of ECM and FA signaling in early MR.

**β-AR Signaling Is Not Altered in MR-Isolated Cardiomyocytes**

Because 1-RB reduced FA signaling loss and prevented p38 MAPK, Hsp27, and AKT activation, we thought that alteration in β-AR signaling may occur in cardiomyocytes at this early stage of MR. To test this hypothesis, myocytes were isolated from LV of control and 4W-MR dogs and plated on laminin-coated dishes for 6 hours in 5% FBS DMEM. Myocytes were then switched to serum-free medium for 1 hour before their treatment with isoproterenol (a nonspecific 1- and 2-AR...
agonist) in the presence or absence of β1-AR antagonist CGP20712A or with a selective β2-AR agonist zinterol. In control-derived myocytes, isoproterenol induced FAK, p38 MAPK, Hsp27, and AKT phosphorylation that was prevented when cells were pretreated with CGP20712A. The β2-AR agonist zinterol also induced an increase in FAK, p38 MAPK, Hsp27, and AKT phosphorylation, suggesting that the activation of these kinases is induced by both β1- and β2-AR stimulation (Figure 7A). Similarly, exposure of myocytes-derived from 4W-MR dogs with β1- or β2-AR agonists led to FAK, p38 MAPK, Hsp27, and AKT phosphorylation, and there were no significant quantitative or qualitative differences observed between these myocytes and myocytes derived from control animals (Figure 7B and 7C). Taken together, the effect of β1-RB in preventing FA signaling loss and increasing p38 MAPK/Hsp27 and AKT activation in LV tissue extracts cannot be explained by an intrinsic alteration in β1- or β2-AR–induced downstream signaling based on these in vitro studies of isolated cardiomyocytes.

**Discussion**

We have previously shown that induction of MR for 4 weeks in the dog was associated with a marked increase in catecholamine release into the LV interstitial fluid space that is significantly attenuated by β1-RB started 24 hours after MR induction.1,2,7 The present study extends this work using samples from these same animals and demonstrates that decreased ECM accumulation in 4W-MR dogs is associated with alteration in FA signaling. FAK tyrosine phosphorylation and FAK association with p130Cas and paxillin is decreased in MR dogs. Interestingly, β1-RB prevented these signaling alterations without improving LV remodeling or completely restoring ECM accumulation. These results suggest that mechanical factors per se are not the trigger for FA signaling changes. Rather, increased adrenergic drive and subsequent stimulation of β1-AR is the main trigger leading to impaired FA signaling early in the course of isolated MR.
In this early stage of isolated MR, loss of FAK tyrosine phosphorylation was associated with the loss of FAK interaction with p130Cas and paxillin, which are important docking sites for other signaling molecules that play an additional role in survival signaling. This is consistent with FAK involvement in the tyrosine phosphorylation of p130Cas and paxillin and indicates that MR induces downregulation of FA signaling, as well as destruction of the FA complex. This is in stark contrast to the activation of FA signaling reported in the early compensated stages of experimentally induced pressure overload in vivo or in isolated cardiomyocytes subjected to pulsatile mechanical.
Figure 7. Preservation of β-AR signaling in MR-isolated cardiomyocytes. Cardiac myocytes were isolated from control (A) or 4W-MR (B) LV as described in Materials and Methods. A and B, Representative immunoblots showing accumulation of phospho-FAK, -p38 MAPK, -Hsp27, and -AKT after treatment with 10 μmol/L isoproterenol (Iso) or 1 μmol/L zinterol (Zint) for 5 minutes compared with control (Ctrl): 1 μmol/L CGP20712A was added 30 minutes before isoproterenol stimulation. C, Summary graphs represent quantitative data from 4 independent experiments from cells derived from 4 different animals. *P<0.05 vs control.
Interestingly, the decrease in FAK and paxillin tyrosine phosphorylation was not associated with their cleavage or caspase-3 activation in 2W-MR (supplemental Figure II and data not shown) and 4W-MR (Figures 2A and 5A) LVs, suggesting that dephosphorylation and disruption of the FA complex occurs before caspase-3 activation and FAK/paxillin degradation. In the failing and dilated hearts in which myocyte apoptosis was identified, FA protein (FAK and paxillin) cleavage has been detected and may involve an increase in caspase-3 activity.26

The role of the constitutive phosphorylation of FA proteins in normal cellular function is not fully understood but may be important for maintaining cell survival and FA integrity in the resting state.16,37 In mice with a selective inactivation of FAK in cardiomyocytes, an eccentric cardiac hypertrophy develops with age and even in the face of angiotensin II infusion or induction of pressure overload with transaxial constriction.19 In another study, persistent challenge of similar transgenic mice with transaxial constriction leads to enhanced cardiac fibrosis and cardiac dysfunction in comparison with challenged genetic controls.20 Despite these cardiac structural changes, both studies failed to detect an increase in myocyte apoptosis. This is in contrast to findings in myocyte-restricted deletion of the β1 integrin in adult mouse hearts in which dilated cardiomyopathy and concomitant heart failure were observed.38 These findings in mice, in addition to our present study, showed that impaired FA signaling is associated with the development of eccentric cardiac hypertrophy. Recent studies in cardiomyocytes in vitro have demonstrated that disruption of FAK signaling prevented hypertrophic responses induced by G protein–coupled receptors and promoted cardiomyocyte apoptosis by anoikis.29,30,39

Protection against apoptosis in this early adaptive phase of isolated MR could be explained by an increase in survival signaling pathways ERK1/2, AKT, and Hsp27 activation that counter proapoptotic events resulting from the loss of FA signaling. In addition, there was no increase in JNK phosphorylation, which has been shown to mediate β1-integrin–induced cardiomyocyte apoptosis.40 Surprisingly, β1-RB prevented both AKT and Hsp27 stimulation, implicating β1-AR stimulation in the activation of these survival signaling pathways that are independent of FAK activation. Nevertheless, there is emerging evidence that prolonged activation of some survival signaling pathways may have deleterious effects on cardiomyocyte survival. Herein, induction of an activated Akt1 gene in the mouse heart induced adaptive cardiac hypertrophy in the acute phase and dilated cardiomyopathy in the chronic phase, suggesting that Akt and Akt-dependent signaling pathways are involved in both physiological and pathological cardiac growth.41 These data emphasize that cardiac apoptosis control is multifactorial and that the balance between pro- and antiapoptotic pathways over time may dictate the transition form heart hypertrophy to failure.

In this study, we showed that β1-RB prevented FA signaling loss and p38 MAPK, Hsp27, and AKT activation induced by 4W-MR. However, we could not detect a significant difference in β1- or β2-AR expression by immunoblot of plasma membrane proteins prepared from control- or MR-derived cardiomyocytes (data not shown). A defect in β2-AR downstream signaling is also unlikely because myocytes derived from control and MR hearts showed similar β1- and β2-AR responses in FAK, AKT, p38 MAPK, and Hsp27 phosphorylation. Although these data indicate an intact β2-AR signaling mechanism of cardiomyocytes in vitro, it is noteworthy that cardiomyocytes were isolated from the whole LV and were stimulated with submaximal concentrations of agonists, which may not allow detecting differences in kinase phosphorylation between control- and MR-derived cardiomyocytes. Furthermore, cardiomyocytes were grown on laminin substrate, which may not reproduce the marked loss of ECM in MR hearts in vivo. This is especially important because plating myocytes on laminin substrate, as opposed to glass, selectively reduced β1-AR and enhanced β2-AR regulation of ICa.13 Thus, our short-term in vitro studies in MR-derived cardiomyocytes plated on laminin may negate the loss of ECM in MR hearts with increased adrenergic drive in vivo.

The selective downregulation of FA signaling in the compensated phase of MR was associated by a small amount of LV dilatation and increased LV fractional shortening. This occurs in spite of the renin–angiotensin system and adrenergic drive activation and is in stark contrast to the activation of FA signaling in experimentally induced pressure overload.22,23 In these dogs, β1-RB decreased catecholamine release into the interstitial fluid in response to electric and angiotensin II stimulation2 and restored FA signaling without affecting LV end-diastolic dimension to wall thickness ratio and wall stress indexes. Our data suggest that prolonged and excessive adrenergic drive is a potential mediator of FA signaling alteration early in the adaptive phase of VO. Consistent with these findings, prolonged stimulation of β2-ARs has been shown to promote disruption of β1-integrin signaling in cultured cardiomyocytes and stimulation of β2-integrin signaling was efficient to protect cardiomyocytes against β2-AR–induced apoptosis.14 However, β1-RB did not prevent a marked loss of endocardial collagen, which could explain the failure to improve short-term diastolic remodeling and function. Long-term therapy of VO with β1-AR does improve isolated cardiomyocyte function but has no effect on interstitial collagen loss and LV dilatation and remodeling (preliminary data and elsewhere). The primary loss of myocyte/fibroblast–ECM scaffolding and their interaction offers a new target, in addition to β1-AR, to attenuate excessive adrenergic drive in the VO of MR.

Sources of Funding This work was supported by NIH grant HL76799 (to A.S.), NIH Specialized Centers of Clinically Oriented Research in Cardiac Dysfunction grant P50HL077100 (to L.J.D.), American Heart Association Grant 0430301N (to A.S.), Department of Veteran Affairs (to L.J.D.), and, in part, by a grant from AstraZeneca (to L.J.D.).

Disclosures None.

References


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_Circ Res._ 2008;102:1127-1136; originally published online March 20, 2008;
doi: 10.1161/CIRCRESAHA.107.163642

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTARY INFORMATION

MATERIALS & METHODS

Materials: Monoclonal antibodies to FAK, paxillin and p130Cas used for Western blot analysis were obtained from BD Biosciences, and 4G10 monoclonal antibodies for phosphotyrosine and phospho-FAK (Tyr397) were from Upstate Biotechnology. Polyclonal antibodies for total FAK (used for immunoprecipitation), p38-MAPK, ERK1/2, AKT, and HSP27 were from Santa Cruz Biotechnology. Polyclonal antibodies for phospho-FAK (Tyr576), -FAK (Tyr925), p38-MAPK (Thr180/Tyr182), -ERK1/2 (Thr202/Tyr204), -AKT (Ser473), and -Hsp27 (Ser82) were from Cell Signaling. Polyclonal antibodies for phospho-FAK (Tyr861) and sarcomeric α-actin were from Sigma. All other chemicals were from standard suppliers.

Cardiac myocytes isolation and culture. Myocyte preparation was performed as described previously with some modification. After hearts were removed, a portion of the left ventricle was excised and a branch of the left anterior descending coronary artery was cannulated. The tissue initially was perfused with Tyrode solution; pH 7.4 at 37°C. After 10-15 min, the perfusion was switched to a Ca\(^{2+}\)-free solution. At ~3-5 min, collagenase (0.4 mg/ml; type II, Worthington Biochemical) and BSA (0.5 mg/ml; Sigma) were added and perfusion was continued for another 10-12 min. Digested tissue was sliced away from the subepicardial area, placed in 10 ml of enzyme solution, and swirled. The supernatant was collected, and 10 ml of fresh Ca\(^{2+}\)-free solution with 0.4 mg/ml collagenase and 0.5 mg/ml BSA was added to the slurry and gently bubbled in a water bath maintained at 37°C. Supernatant was collected for six subsequent washes. After
5 min of settling, the final pellet was washed in 10 ml of incubation buffer containing (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO4, 1.2 KH2PO4, 0.68 glutamine, 11 glucose, 20 NaHCO3, 5 HEPES, 5 pyruvate, 10 taurine, and 0.5 CaCl2 plus 2% BSA. After another 30 min of settling, the pellet was washed a second time with incubation buffer now containing 1 mM CaCl2 and was allowed to equilibrate at room temperature for 60 min. Myocytes were plated at a density of ~0.5 × 10^5 cells/cm^2 on plastic culture dishes coated with laminin (10 µg/ml; BD Bioscience). The culture medium consisted of Eagle's minimum essential medium (GIBCO) with the following additions: nonessential amino acids (GIBCO), vitamins (2X; GIBCO); 10 µg/ml insulin (Sigma), 10 µg/ml transferrin (GIBCO); 5% fetal bovine serum, and 100 U/ml penicillin-100 µg/ml streptomycin. Cells were incubated at 37°C in 5% CO2 in a humidified environment for 6 hrs then medium was switched to serum free DMEM/F12 1hr before treatments.

**Immunoprecipitation and immunoblot analysis.** Extraction of proteins from LV or cultured cells was performed as previously described.²³ Lysates were clarified by centrifugation at 12,000 rpm, and the supernatants (1 mg of protein/ml) were subjected to immunoprecipitation with corresponding antibodies. After overnight incubation at 4°C, protein A- or G-agarose beads were added and left for an additional 3 hrs. Immunocomplexes were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by western blot analysis according to methods published previously or to the manufacturer's instructions.²³ Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.
Caspase-3 Assay. Caspase 3 activity was measured with CaspACE assay system (Promega, Madison, WI). In brief, heart lysates were prepared by dounce homogenization in lysis buffer provided with the kit. The lysates were centrifuged at 15,000g for 20 minutes at 4 °C, and the supernatants containing 100 µg protein were used for caspase-3 assay. Caspase-3 activity was examined by measurement of the rate of cleavage of fluorogenic conjugated substrate MCA-Val-Asp-Gln-Met-Asp-Gly-Trp-Lys-(DNP)-NH₂. The specificity of the assay was confirmed by addition of the specific caspase-3 inhibitor Z-DQMD-FMK in the reaction mixture at a concentration of 50 µM during the incubation.

Apoptotic cell death ELISA. Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes fragments associated with apoptotic cell death.

Evaluation of apoptosis in tissue sections. TUNEL labeling using fluorescein-labeled dUTP was performed on 5-µm sections from LV fixed by 10% formalin per the manufacturer’s protocol (In Situ Cell Death Detection kit; Roche Applied Science, Indianapolis, IN). Sections were then counterstained with DAPI for 15 minutes to visualize nuclei. Digital photographs were taken under fluorescence microscopy at ×200 magnification. Twenty-five to thirty random high-power fields from each heart sample were chosen and blindly quantified.

Immunohistochemistry. Frozen Sections from MR and control LVs were fixed in 4% paraformaldehyde/PBS 30 min at 4 °C. After washes in PBS, sections were preincubated with 2% BSA in PBS for 30 min at room temperature, followed by overnight incubation
with the primary anti-FAK polyclonal antibody. After washes in PBS, sections were then incubated with monoclonal anti-α-actin (sarcomeric) antibodies followed by incubations with Alexa-conjugated anti-mouse (-rabbit) Ig antibodies. After 2 washes in PBS, cells were incubated with DAPI for 30 min at room temperature, mounted, and then examined using an epifluorescence microscope.

Data Analysis. All data are presented as mean±SEM. One-way repeated-measures ANOVA. A value of $P<0.05$ was considered significant

SUPPLEMENTARY RESULTS

LV remodeling and cardiac function in 2W-MR and 2W-MR+$\beta_1$-RB animals.

Hemodynamics.

After 2 weeks of MR, mean arterial pressure, heart rate, and cardiac output were significantly decreased compared with controls, whereas LV end-diastolic pressure and mean systemic vascular resistance did not significantly differ from controls in the untreated 2W-MR dogs. There was a trend toward increases in LV mass in 2W-MR and 2W-MR+$\beta_1$-RB dogs but this did not achieve statistical significance. $\beta_1$-RB had no significant effect on cardiac output, heart rate, LV end-diastolic pressure, and mean systemic vascular resistance compared to untreated 2W-MR (Supplemental Table S1). However, there was a significant increase in LV end-diastolic pressure in 2W-MR+$\beta_1$-RB dogs compared to normal animals.

Echo LV remodeling and function
Compared with baseline both 2W-MR and 2W-MR+β₁-RB groups had a similar increase in LV end-diastolic dimension compared to baseline (Supplemental Table S1). In addition, cardiac function assessed by LV fractional shortening was significantly increased from baseline in 2W-MR group, as LV end-systolic dimension remained unchanged (Supplemental Table S1). However, this increase was not affected in 2W-MR+β₁-RB group. Eccentric LV remodeling, as measured by LV wall thickness/end-diastolic dimension was decreased similarly in 2W-MR and 2W-MR+β₁-RB group.

REFERENCES


ONLINE FIGURES & TABLES
Supplemental Table S1: LV hemodynamics and echocardiography in control, 2W-MR, and 2W-MR+ β1-RB dogs

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>2W-MR (n=6)</th>
<th>2W-MR+β1-RB (n=6)</th>
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</thead>
<tbody>
<tr>
<td><strong>Heart rate, bpm</strong></td>
<td>94 ±6</td>
<td>76 ±4*</td>
<td>74 ±2*</td>
</tr>
<tr>
<td><strong>Mean arterial pressure, mm Hg</strong></td>
<td>84 ±3</td>
<td>64 ±7*</td>
<td>65 ±4*</td>
</tr>
<tr>
<td><strong>LV end-diastolic pressure, mm Hg</strong></td>
<td>4 ±1</td>
<td>9 ±2</td>
<td>15 ±3*</td>
</tr>
<tr>
<td><strong>Systemic vascular resistance, dyne · s · cm⁻¹</strong></td>
<td>2007 ±219</td>
<td>2620 ±235</td>
<td>3123 ±517</td>
</tr>
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<td><strong>Cardiac output, L/min</strong></td>
<td>3.6 ±0.4</td>
<td>2.1 ±0.01*</td>
<td>1.9 ±0.5*</td>
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<tr>
<td><strong>LV mass, g/kg</strong></td>
<td>4.4 ±0.2</td>
<td>4.8 ±0.3</td>
<td>4.8 ±0.3</td>
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<tr>
<td><strong>LV end-diastolic dimension, % change</strong></td>
<td>...</td>
<td>14.5 ±4.7†</td>
<td>18.2 ±2.4†</td>
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<tr>
<td><strong>LV end-systolic dimension, % change</strong></td>
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<td>0.8 ±4.8</td>
<td>-6.9 ±3.9</td>
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<tr>
<td><strong>LV end-diastolic wall thickness, % change</strong></td>
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<td>-5.1 ±4.3</td>
<td>-11.7 ±3.2</td>
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<tr>
<td><strong>Wall thickness/end-diastolic dimension, % change</strong></td>
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<td>-15.9 ±5.7†</td>
<td>-25.0 ±3.8†</td>
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<tr>
<td><strong>Fractional shortening, % change</strong></td>
<td>...</td>
<td>22.7 ±6.3†</td>
<td>43.6 ±11.0†</td>
</tr>
</tbody>
</table>

Values presented as mean±SEM. *P<0.05 vs Normal Dogs. †P<0.05, % change vs baseline study.

Supplemental Figure S1

Volume percent collagen of LV endocardium and epicardium in control, 2W-MR, and 2W-MR+β1-RB dogs. *P<0.05 vs control.

Supplemental Figure S2
Left ventricular extracts from control, 2W-MR, and 2W-MR+β₁-RB dogs were immunoprecipitated (IP) with anti-FAK antibodies and immunoblotted with anti-phosphotyrosine antibodies. Top, representative autoradiogram (with each lane from a single gel exposed for the same duration). Bottom, fold induction, n=6 each group, *P<0.05 2W-MR vs. control.

Supplemental Figure S3
LV sections from control (A, C, E) and MR (B, D, F) dogs double stained with anti-FAK (green) and sarcomeric α-actin (red) antibodies. Nuclei were visualized with DAPI staining (Blue). FAK labeling was localized around cardiomyocytes with some staining of the interstitial space. No qualitative change in FAK distribution was observed between control and MR LVs. Bar represents 50 µm.

Supplemental Figure S4
Top, representative immunoblot showing accumulation of phospho-Pyk2 Tyr-402 in LV extracts from control, 2W-MR, and 2W-MR+β₁-RB. Blot was stripped and blotted with anti-Pyk2 antibodies. Bottom, fold induction, n=6 each group.

Supplemental Figure S5
Top, representative immunoblots showing accumulation of phospho-ERK₁/2 in LV extracts from control, 2W-MR, and 2W-MR+β₁-RB. Blots were stripped and blotted with anti-ERK₁/2 antibodies. Bottom, fold induction, n=6 each group, *P<0.05 2W-MR vs. control.
Supplemental Figure S1
Supplemental Figure S2

FAK phosphorylation (fold increase over control)

- Ctrl
- 2W-MR
- 2W-MR + β1-RB

P-Tyr and FAK bands from IP: FAK experiment.
Supplemental Figure S3
Supplemental Figure S4
Supplemental Figure S5

ERK2 phosphorylation (fold increase over control)